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14. ABSTRACT

The deregulation of cell cycle checkpoints, with loss of regulation at the G1/S transition, has been shown to play an important role in the transformation to a malignant phenotype. Our studies have focused on cyclin E, which appears in late G1 and flanks the restriction point .We hypothesize that alterations of cyclin E in ovarian cancer cells contributes to the oncogenesis of ovarian tumors and negatively impacts outcome in patients with Stage I-III cancer. In this proposal we will i) develop a comprehensive ovarian cell line model for characterization of the role of cyclin E in ovarian cancer, ii) delineate the role of cyclin E and its tumor specific LMW forms in the development of malignant phenotype in vitro and in nude mice. iii) establish the prognostic value of the hyperactive forms of cyclin E in patients with Stage I-III ovarian cancer and iv) examine the biochemical significance of the LMW forms of cyclin E in tumor specimens. The results from our studies will provide much needed information about the molecular biology of ovarian carcinoma and may open new avenues for the development of targeted therapies.

15. SUBJECT TERMS
Ovarian cancer, cyclin E, LMW forms, predictive marker

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Introduction:

The overall purpose of this 3 year study is to test the hypothesis that alterations of cyclin E in ovarian cancer cells contributes to the oncogenesis of ovarian tumors and negatively impacts outcome in patients with Stage I-III ovarian cancer.

Normal cell division is precisely regulated by checkpoints at distinct junctures in the cell cycle (1). The G1/S checkpoint appears to be the most relevant to the process of carcinogenesis and is invariably deregulated in human tumors (2). Cells in G1 are normally responsive to extracellular mitogenic stimulation that drives them into quiescence or into another round of proliferation (1). In cancer cells, this dependence on exogenous signals is uncoupled through a potential myriad of mutations thereby preventing the cells from exiting the cell cycle (2). Central to the passage of cells through G1 into S phase is the Rb pathway (3). This pathway is positively regulated through sequential phosphorylation of Rb by cyclin dependent kinases (cdks) and their associated cyclins.

Human cyclin E was first identified in 1991 through screening of human cDNA libraries for genes that would substitute for G1 cyclin mutations in yeast (4, 5). In normal cells, the transcription and degradation of cyclin E are tightly regulated thereby ensuring the periodicity of cyclin E to the G1 window (6). Several laboratories, including ours, have demonstrated a link between altered expression of cyclin E and oncogenesis (7, 8). Using a breast cancer model we have shown alterations in regulation and expression of cyclin E at several levels: i) amplification of the cyclin E gene, ii) overexpression of cyclin E mRNA, iii) constitutive expression of cyclin E protein throughout the cell cycle and iv) presence of hyperactive LMW forms of cyclin E that

more readily induce G1/S transition (9-12). These changes in the regulation of cyclin E lead to constitutive expression and activity throughout the cell cycle.

The most significant alteration in the regulation of cyclin E is the generation of LMW forms of cyclin E in tumor, but not normal epithelial cells. Our studies suggest that these hyperactive LMW forms are generated by post-translational modification of the full length cyclin E by the elastase class of serine proteases, which cleaves the full length protein at specific sites at the amino terminus generating low molecular weight isoforms (6, 10) . In addition to the full-length protein at 50-kDa (EL-1), we have identified 5 LMW forms (EL2 through EL-6). EL-4 appears to be the result of alternative translation at methionine 46. The remaining 4 of the 5 LMW forms in tumor cells are accounted for by elastase mediated proteolytic cleavage at 2 domains in cyclin E, which results in 2 pairs of closely migrating doublets (EL-2/EL-3 and EL-5/EL-6). These isoforms are biochemically hyperactive, as evidenced by their enhanced ability to phosphorylate histone H1 and GST-Rb, compared to full length cyclin E (10). Transfection of the LMW isoforms into normal mammary epithelial cells has significant mitogenic effect, readily inducing cells to enter the cell cycle (10).

The biologic importance of cyclin E is supported by clinical observations of prognosis in a number of human tumors. In breast cancer, we have reported that the presence of the LMW forms in breast cancer patients is associated with increasing grade and stage as well as significantly worse prognosis (13, 14). In ovarian cancer patients, high cyclin E expression has been associated with low overall survival rates (15). Even in patients with advanced, suboptimally debulked ovarian epithelial cancers, high cyclin E expression has remained an independent poor prognostic factor (16). Cyclin E overexpression has similarly been linked to

adverse outcomes in patients with gastric (17), bladder (18) and non-small cell lung carcinomas (19-21).

Results

In the first 2 years of the proposed grant we have completed the first 3 aims of this grant application and have published our work in the Journal Oncogene (22).

Task 1: Characterization of normal ovarian epithelial cell lines and ovarian cancer cell lines. Cyclin E is deregulated in epithelial ovarian tumors In order to determine whether cyclin E is frequently overexpressed in ovarian epithelial cancers, a panel of such tumors was obtained from the Gynecologic Oncology Tumor Bank at M.D. Anderson Cancer Center and expression of FL and LMW cyclin E assessed by Western blot analysis. A representative panel of samples is shown in Figure 1.

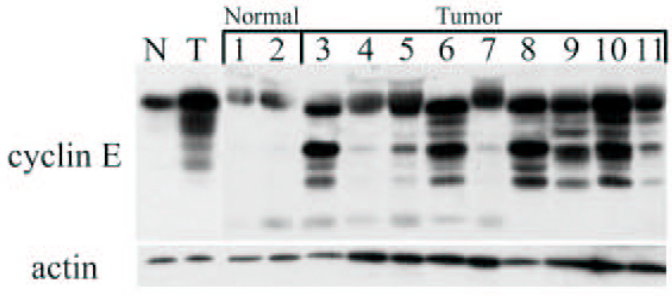

Figure 1: Western blot analysis of cell cycle regulators in human ovarian tumor samples. Protein extracts were analyzed on Western blots (50 µg of protein extract/lane) and hybridized with the indicated antibodies. Whole cell lysates extracted from 2 normal ovaries (lanes1, 2) and 9 ovarian cancer cases (3-11). Lanes 3-5 are low grade, stage IIIc tumors, while lanes 6-11 are high grade, stage IIIc and IV tumors. The control lanes correspond to cultured normal (N) and tumor (T) cell lines.

Of the 25 tumor samples examined, total cyclin E expression was increased in 17 (68%) samples compared to normal ovarian tissue. In all 17 cases, overexpression of cyclin E was due to the presence of the LMW isoforms of cyclin E. Consistent with previous data from breast cancer patients {Keyomarsi, 1994 #782; Keyomarsi, 2002 #783}, the incidence of cyclin E

overexpression in these ovarian tumor samples appeared to correlate with grade and stage of disease.

Tumors with cyclin E over-expression have high kinase activity: To examine the biochemical properties and protein complex interactions of cyclin E in these tumors tissue samples, 6 samples were selected for further analysis (Figure 2) - 3 with normal cyclin E expression (samples: 01-018, 00-066, 00-273) and 3 with expression of the LMW isoforms of cyclin E (samples: 00-140, 00-285, 00-007). The expression of cyclin E, cdk2, p21 and p27 in these tumor samples is shown in Figure 2A. Of note, nearly all these samples had high p27 expression; p21 levels were higher in the tumor samples with LMW cyclin E expression. Despite high levels of expression of the CKIs, the cyclin E kinase activity, as measured by phosphorylation of Histone H1, was 3-fold higher in the tumor samples that expressed LMW cyclin E (Figure 2B).

We next sought to determine whether the differences in cyclin E kinase activity may be related to differences in binding of the CKIs to FL and LMW cyclin E (Figure 2C). The tumor cell lysates were subjected to 2 rounds of immunoprecipitation with p27 in order to ensure depletion of all p27 protein from the sample (Figure 2C, last panel) and subsequently examined by western blot analysis with the antibodies indicated. Immunoblotting with antibody to cyclin E confirmed that the LMW isoforms in tumor samples readily bind to p27 (Figure 2C, IP: p27; WB: cyclin E). Expression of cdk2 was similar in p27-FL and p27-LMW complexes. Data from these patient derived tumor samples demonstrate that in vivo, the expression of LMW isoforms of cyclin E results in an increase in the biochemical activity of the cyclin E-cdk2 kinase complex despite high levels of CKIs and effective binding of the LMW isoforms to p27.

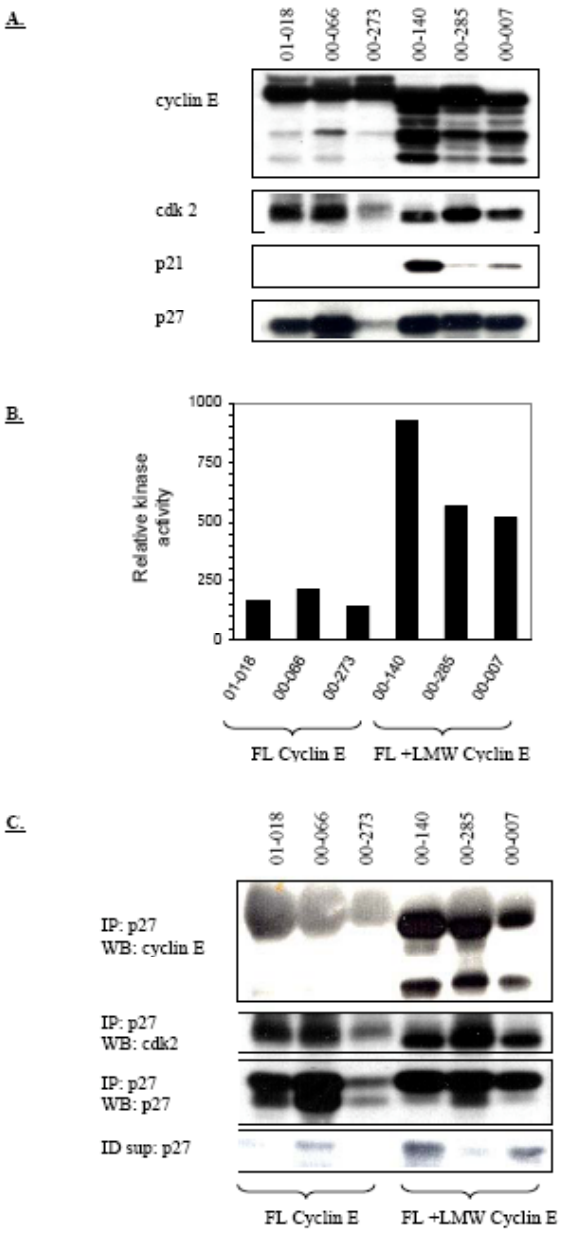

Figure 2: LMW cyclin E overexpression in ovarian carcinoma samples correlates with increase kinase activity despite high level of binding to p27. Protein extracts from 3 LMW cyclin E overexpressing tumors (00-140, 00-285, 00-007) and 3 tumors that express only FL cyclin E (01-018, 00-066, 00-273) were characterized by western blot analysis (A), cyclin E kinase assay (B) and p27 immunoprecipitation assay (C). Protein extracts were analyzed on Western blots (50 µg of protein extract/lane) and hybridized with the indicated antibodies (A). For kinase assay (B), lysates (300mg protein) were immunoprecipitated with polyclonal anti-cyclin E antibody/sepharose A beads, then incubated with kinase reaction buffer containing 5mg histone and samples electophoresed on a 13% SDS-PAGE gel. The gel is stained, destained, dried and bands corresponding to Histone H1 were excised and quantified by Cerenkov counting. To determine whether p27 was bound to LMW cyclin E, 300mg of protein extract was immunoprecipitated with polyclonal anti-p27/sepharose A beads (C), then electrophoresed on a SDS-PAGE gel and probed with monoclonal antibody indicated. Western blot analysis of the immunodepleted supernatants (ID sup) confirm near complete depletion of p27 from these samples.

Task 2: *In vitro* transfection and generation of stable cyclin E overexpressing clones and

Task 3: Characterize *in vitro* the oncogenic potential of cyclin E/LMW expressing clone

MDAH 2774 T1 clones have altered biologic properties: In order to directly assess the biologic and biochemical role of the cyclin E overexpression seen in ovarian carcinoma specimens, we developed an *in vitro* model of cyclin E overexpression, using the MDAH 2774 cell line, which expresses only the FL cyclin E protein. As described above, overexpression of cyclin E in ovarian tumor tissues was due to the presence of the LMW isoforms of cyclin E,

therefore we generated stable clones of MDAH 2774 to express one of the pair of LMW doublets, trunk 1 (T1) (Figure 3A). Two different clones of vector alone (3.1a6, 3.1b3) and T1 (T1a5 and T1a7) were selected and used for these experiments. These T1 clones have high expression of the EL2/3 doublet, stable over many passages (data not shown). Although cyclin A levels appear to be increased and p27 levels decreased in the T1 clones, these findings likely represent clonal variation since similar changes are seen in one of the empty vector clones (3.1a6) . No alteration was seen in expression of cyclin D1, cdk2 and p21 as a result of transfection with T1 cDNA.

Consistent with the data from tumor samples, we noted at least a 10-fold increase in cyclin E associated kinase activity in the T1 clones as measured by phosphorylation of Histone H1 (Figure 3B). Compared to parental MDAH 2774 cells and empty vector clones, the T1 clones had altered biological properties (Figure 3C). When harvested from subconfluent cultures and assessed for cell cycle profile by flow cytometry, both T1 clones had a significant increase in S-phase fraction of approximately 20% compared to parental MDAH-2774 cells (T1a5, 22.2%±5.25; T1a7, 20.7%±6.41, $p=0.02$). Growth kinetics demonstrated exponential growth rates for all cell lines (parental, empty vector and T1 clones). However, doubling times calculated from the start of assay to end of the exponential growth phase for each cell line showed a 10-15% decrease in doubling time in the T1 clones (T1a5, -10%±2.82; T1a7, -13%±3.09, $p<0.05$). This increase in S-phase with concomitant decrease in doubling times is consistent with increase in the proliferative fraction as a result of deregulation of G1/S by overexpression of T1. In clonagenic assays, T1 cells plated at low density (100 cells/plate) had increased capacity to form colonies ≥ 2mm after 13 days of culture (T1a5, 19.6%±13.64; T1a7, 19.9%±15.16, $p<0.05$). The changes in S-phase fraction, doubling time and clonagenic assay

seen with the T1 clones were all statistically significant as compared to parental cell lines ($p<0.05$).

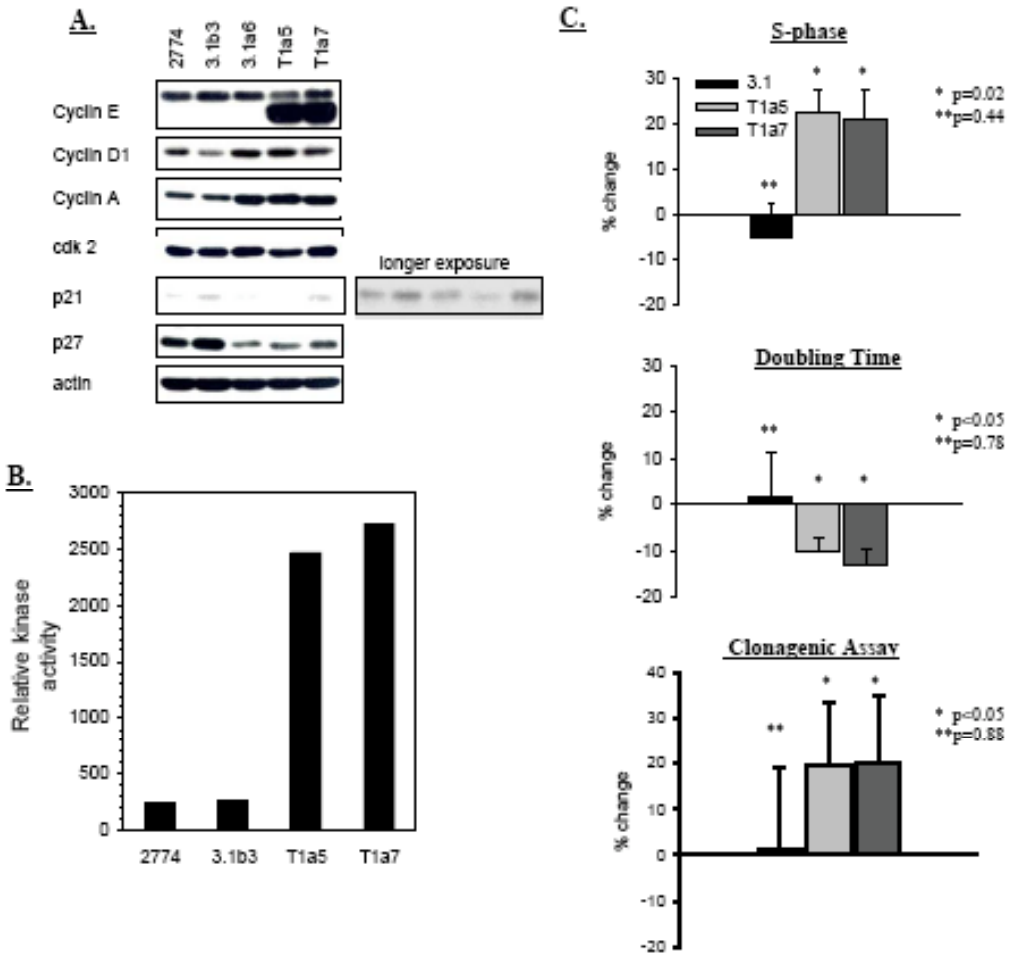

Figure 3: MDAH-2274 clones overexpressing LMW cyclin E (T1) have altered biologic and functional properties. (A) Western immunoblot profile of parental cells (2774), empty vector clones (3.1b3, 3.1a6) and T1 clones (T1a5, T1a7). Subconfluent cultures of MDAH-2774, 3.1 and T1 clones were harvested, lysed and protein extract obtained. Protein extracts then analyzed on Western blots (25 µg of protein extract/lane) and hybridized with the indicated antibodies. For p21, baseline levels in all cell lines were low; delayed exposure required to visualize expression. (B) Lysates (300mg protein) from indicated cell lines were immunoprecipitated with polyclonal anti-cyclin E antibody/sepharose A beads, then incubated with kinase reaction buffer containing 5mg histone and samples electophoresed on a 13% SDS-PAGE gel. The gel is stained, dried and bands corresponding to Histone H1 were excised and quantified by Cerenkov counting. (C) For S-phase determination, cells were plated at low density in150mm plates. After 42-48hours, and at a confluency of no greater than 50%, cells were harvested and processed for flow cytometry. Data is average of 3 experiments and expressed as percentage change relative to MDAH 2774 cells (*p=0.02, **p=0.44). Growth kinetics were graphed logarithmically and doubling time was calculated during the exponential (i.e linear) growth phase. Data is average of 6 wells/timepoint and expressed as percentage change relative to MDAH 2774 (*p<0.05, **p=0.78). For clonagenic assays, cells were plated at low density (100cells/plate) in 100mm dishes. At 13 days, the cells were fixed and stained with crystal violet in 100% ethanol suspension. Plates were scored for the number of visible colonies ≥2mm and data expressed as percentage change relative to MDAH 2774 (*p<0.05, **p=0.88).

MDAH 2774 T1 clones are resistant to lovastatin mediated G1 arrest: To determine the effect of cyclin E overexpression on the G1/S checkpoint, we tested the ability of the T1 clones to arrest in G1. We have previously shown that lovastatin leads to G1 arrest by inhibition of the proteosome and subsequent increase in p21 and p27 levels {Rao, 1998 #803; Rao, 1999 #804; Gray-Bablin, 1997 #802}. Cells were treated with escalating doses of lovastatin and cell cycle distribution assessed by flow cytometry (Figure 4A). Our results showed that compared to parental MDAH 2774 cells and empty vector (3.1) clones, a 3-fold higher dose of lovastatin was required to achieve a 50% reduction in the number of MDAH 2774 T1 cells entering S-phase (T1 6mM lovastatin vs parental 1.5mM lovastatin). In addition, even at the highest dose of lovastatin (24mM), and in contrast to the parental and empty vector cells, the T1 clones did not completely arrest in G1 (Figure 4A) and in fact reached a plateau that persisted to the highest doses used (24mM). We also examined the cyclin E associated kinase activity in lovastatin treated parental, empty vector and T1 clones using Histone H1 as substrate. Our results reveal that while the parental and vector alone controls were exquisitely sensitive to lovastatin, with kinase activity plummeting by 6mM of drug, the kinase activity in T1 clones was minimally affected by lovastatin treatment throughout the concentration range used (Figure 4B).

We next examined if the differential flow cytometry and kinase activity profiles of lovastatin treated MDAH 2774 and T1 clones would result in altered expression of key G1/S regulators (Figure 4C). Our Western blot analysis revealed that in parental MDAH 2774 cells and empty vector (3.1) controls, the phosphorylated forms of pRb rapidly disappeared and were completely undetectable by 3mM (Figure 4C, compare lanes 1 and 7 to 3 and 8 respectively), consistent with the suppression of kinase activity and induction of G1 arrest (Figure 4A). In contrast, in the T1 clones, although the absolute level of phosphorylated pRb diminished with lovastatin treatment,

it remained present to the highest dose of drug used (Figure 4C, compare lanes13 to 18). This level of phosphorylated pRb was sufficient to maintain kinase function (Figure 4B) thereby substantially preserving the proliferative fraction of T1 clones (Figure 4A). These differences in pRb phosphorylation were seen despite a similar level of induction of p21 and p27 in parental, 3.1 and T1 clones; the higher levels of p27 in the 3.1 cells are seen at baseline (Figure 3) and likely represent clonal variation in the expression of this CKI. Cyclin E and cdk2 levels were not affected by lovastatin treatment in any of the clones.

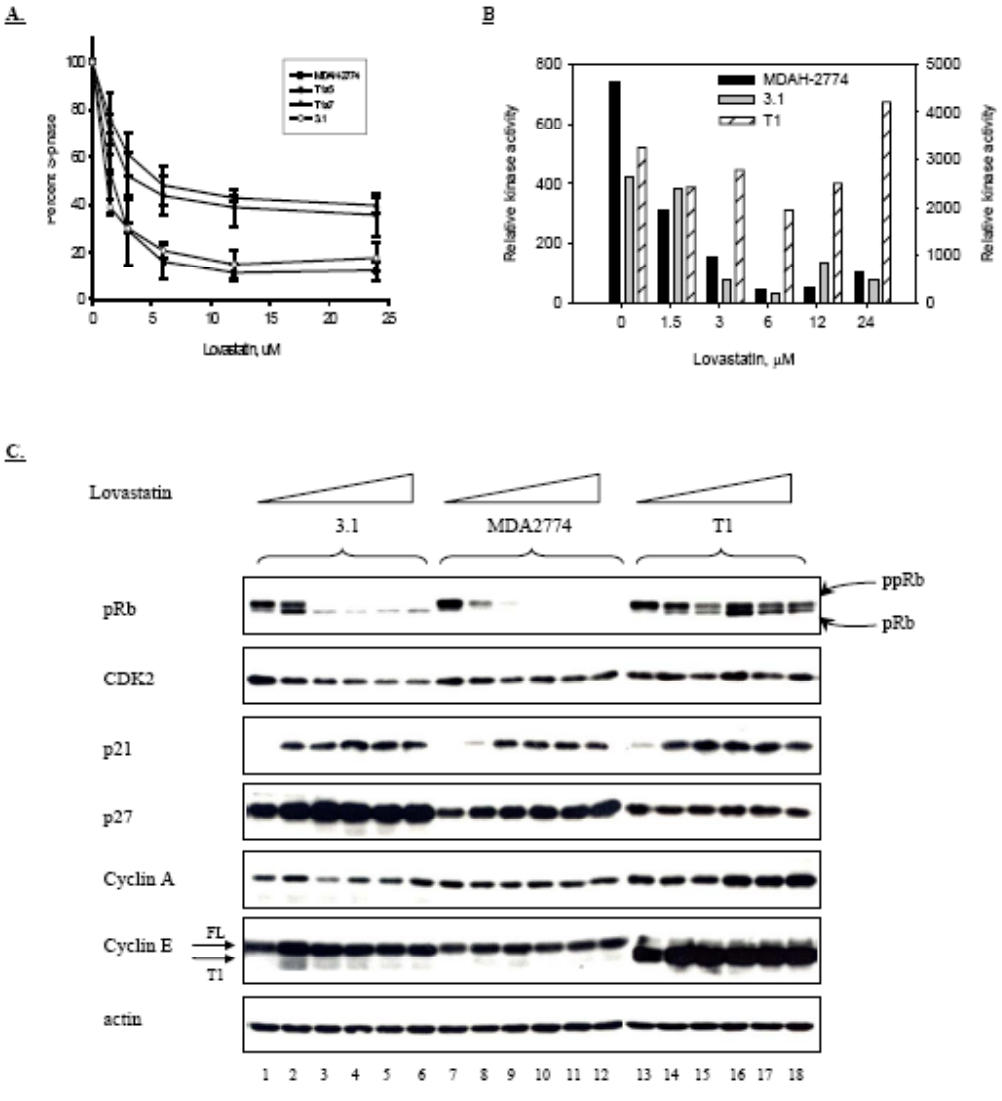

Figure 4: MDAH-2774 T1 clones are resistant to G1 arrest and maintain cyclin E/cdk2 kinase function despite induction of p21 and p27 with lovastatin treatment. (A) Individual lines were treated with lovastatin for 48-72 hours then harvested for cell cycle analysis by flow cytometry. Data was normalized relative to untreated control samples. (B) Lysates (200mg protein) prepared from lovastatin treated cells were immunoprecipitated with polyclonal anti-cdk2 antibody/sepharose A beads, then incubated with kinase reaction buffer containing 5mg histone and samples electophoresed on a 13% SDS-PAGE gel. The gel is stained, destained, dried and bands corresponding to Histone H1 were excised and quantified by Cerenkov counting. Data is average of 2 experiments. Relative kinase activity of parental MDAH-2774 cells and empty vector clones represented on the Y-axis on the left; relative kinase activity scale on right represents counts obtained for the T1 clones. (C) Protein extracts were analyzed on Western blots (25 µg of protein extract/lane) and hybridized with the indicated antibodies.

LMW cyclin E isoforms in MDAH-2774 clones bind p21 and p27: In order to assess if resistance to lovastatin mediated G1 arrest in cyclin E-T1 clones was due to lack of binding of p21 and p27 to the LMW cyclin E isoform we next undertook immunoprecipitation assays to examine the binding of the CKIs to cyclin E-T1 (Figure 5). Cell lysates from lovastatin treated MDAH-2774, empty vector (3.1) and cyclin E-T1 clones were immunoprecipitated with either p27 or p21 and the immunoprecipitates subjected to western blot analysis with the indicated antibodies (Figure 5A and B). We found that the LMW forms of cyclin E bind to p21 and p27 and that this binding is increased by increasing doses of lovastatin treatment. Of interest, FL cyclin E appears to bind with greater affinity to p27 compared to p21 with little, if any, FL cyclin E detected when p21 immunoprecipitates were subjected to immunoblot with the cyclin E antibody (Figure 5B). In contrast, the LMW forms of cyclin E appear to bind strongly to both p21 and p27; cyclin E immunoblotting of p21 and p27 immunoprecipitates revealed significant expression of the cyclin E-T1 protein in both p21 and p27 complexes. In addition, within the sensitivity limits of western blot analysis, the p21 complexes did not appear to bind detectable amounts of cdk2.

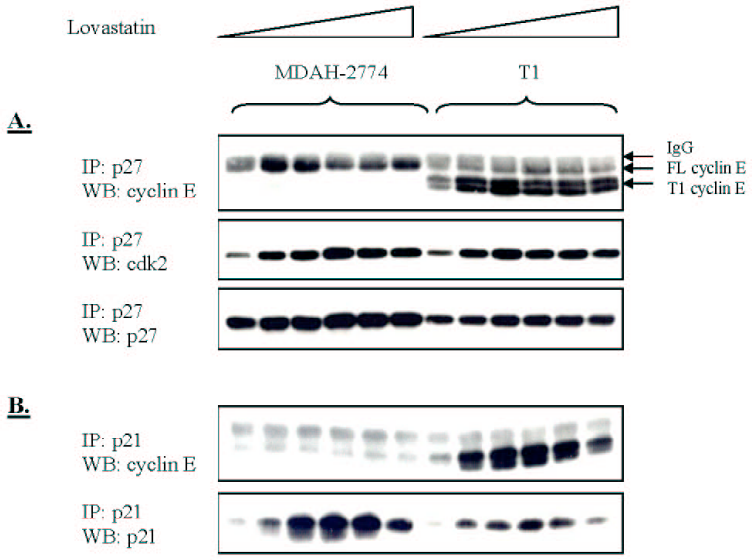

Figure 5: LMW cyclin E binds to p21 and p27. Lysates (200mg protein) obtained from lovastatin treated MDAH-2774 and MDAH-2774 T1 clones were immunoprecipitated with polyclonal p27/sepharose A beads (A) or polyclonal p21/sepharose A beads (B), then electophoresed on a SDS-PAGE gel and probed with monoclonal antibody indicated. No cdk2 binding was observed in the p21 immunoprecipitates.

These immunoprecipitation assay results confirm the robust binding of the LMW cyclin E isoforms to p21 and p27, but suggest that such binding to the CKIs does not result in significant inhibition of cdk2 function (Figure 4B) or abrogation of pRb phosphorylation (Figure 4C). Additionally, these *in vitro* cell line data results validate our findings in ovarian tumor tissue samples (Figure 2).

MDAH 2774 T1 clones are more sensitive to cisplatin cytotoxicity*:* Given the increase in S-phase population as a result of T1 overexpression, we next examined if such a shift in cell cycle distribution would increase the sensitivity of T1 clones to chemotherapeutic agents known to target the S-phase. Cisplatin was chosen for these studies as this is commonly used in the treatment of ovarian cancer and ovarian tumor response to cisplatin has been correlated with proliferative rate {Itamochi, 2002 #1004}. We sought to directly test this hypothesis in our isogenic model (Figure 6). In preliminary dose escalation and time course MTT assays, 1 mg/ml of drug for a 72 hour period of treatment was found to have maximal effect in our system (Figure 6A and data not shown). At 72 hours of treatment with cisplatin, the T1 clones were clearly more sensitive to drug effect at all doses examined (Figure 6A). Next, we studied the growth effects of cisplatin over a prolonged period. For this analysis, cells were treated with 1mg/ml of cisplatin for 72 hours, then the drug was removed and the cells allowed to recover. Final cell proliferation was analyzed by MTT assay at 0, 2, 4 and 8 days after cisplatin removal. As seen in Figure 6B, at all time points examined, T1 clones were more sensitive to cisplatin treatment compared to empty vector clones. Growth recovery in the 3.1 clones was observed 4 days after removal of cisplatin with rapid recovery thereafter such that at 8 days after drug removal, the growth rate of the treated empty vector cells approached that of untreated controls. In contrast, even 8 days after removal of cisplatin, there was no notable recovery of the T1 clones.

To determine whether the differential effect of cisplatin on T1 and 3.1 clones were secondary to growth inhibition or due to increased cytotoxicity, we performed clonagenic assays (Figure 6C). Cell lines were plated at densities of 100, 500 and 1000 cells/plate, treated with cisplatin for 72 hours, then maintained in culture for a total of 2 weeks. Colonies were enumerated at the end of 2 weeks by staining with crystal violet. We found that at all doses of cisplatin examined, T1 clones were significantly more susceptible to kill compared to the empty vector clones ($p<0.05$)(Figure 6C). The calculated IC_{50} for both T1 clones, the 3.1b3 and the 3.1a6 clones were 0.32mg/ml, 0.44mg/ml and 0.56mg/ml respectively. Hence the T1 clones were nearly twice as sensitive to cisplatin treatment than empty vector clones. These results corroborate in a stable, isogenic model that cyclin E deregulation in ovarian carcinoma results in changes in proliferative rate that may be exploited for potential therapeutic effect.

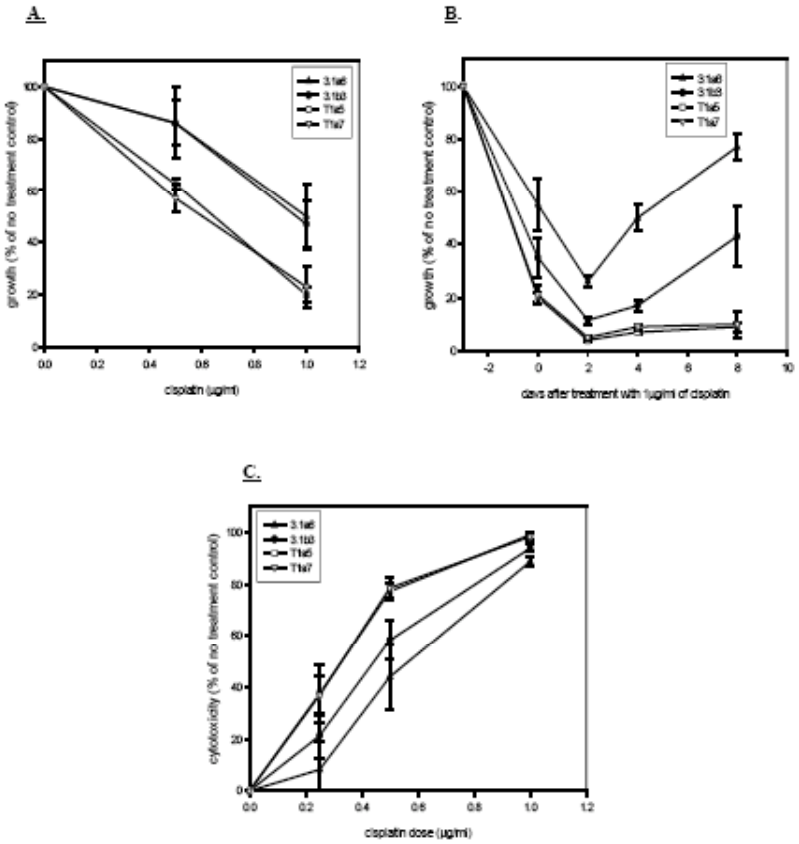

Figure 6: MDAH-2774 T1 clones are more sensitive to cisplatin treatment. (A,B) 3.1 empty vector and T1 clones plated in 96 well plates at 1000 cells/ well. After 24 hours, cells were treated with cisplatin at doses indicated for 72 hours. Growth inhibitory effects tested by MTT assay either at 72 hours (A) or at timepoints indicated after removal of drug (B). Media was changed every other day following removal of drug. (C) Cell lines plated at 100, 500 or 1000 cells/plate. After 24 hours, cisplatin added at doses indicated for 72 hours of treatment. Drug then removed and cells maintained in culture for a further 10 days prior to analysis. T1 clones more susceptible to cisplatin cytotoxicity compared to 3.1 clones at all doses tested ($p<0.05$).

DISCUSSION

We have previously described the generation of the LMW isoforms of cyclin E as a tumor-specific event that results in the post-translational modification of the full length protein into 2 pairs of lower molecular weight proteins {Harwell, 2000 #784; Porter, 2001 #785}. Previously, in normal mammary epithelial cells, we have seen that these LMW forms are biochemically more active than the full length forms and more efficient in mediating the G1/S transition {Porter, 2001 #785}. However, the biologic consequences of cyclin E overexpression in tumors has not been fully studied. Of interest, by using western blot assays to resolve FL and LMW cyclin E, we have found that cyclin E overexpression in tumor cells is predominantly secondary to the acquisition of the LMW isoforms; rarely is full length cyclin E overexpession seen in the absence of the LMW isoforms (Figure 1, {Keyomarsi, 2002 #783} and data not shown). Therefore, we sought to determine the impact of overexpression of LMW cyclin E in tumor cells. In our current ovarian tumor model we find that overexpression of LMW cyclin E confers a number of important biologic properties, including an increase in S-phase, an increase in the ability to form colonies and resistance to G1 arrest.

The finding that deregulation of the G1/S checkpoint in a tumorigenic cell line results in further acquisition of a more biologically aggressive phenotype may help explain clinical observations made regarding cyclin E expression in tumor cells. The incidence of cyclin E deregulation appears to increase with increase in grade and stage of tumor {Keyomarsi, 1994 #782}. In addition, cyclin E overexpression, even in advanced stages portends a poorer outcome (Farley et al., 2003; Keyomarsi et al., 2002). These clinical observations suggest that cyclin E deregulation offers additional biologic properties that enhance the aggressive phenotype even late in the

oncogenic process; our data suggests that these additional biologic properties include an increase in proliferative capacity and a resistance to G1 arrest. Although not the primary focus of this study, we have previously shown that deregulation of cyclin E in cancer cell lines results in constitutive expression of this protein throughout the cell cycle (Keyomarsi et al., 1995). Therefore the possibility that deregulated cyclin E may impact the S and G2/M checkpoints with further gain of function cannot be excluded.

Hyperactivity of the cyclin E/cdk 2 kinase complex appears to underlie these altered biologic properties of tumor cells transfected with the LMW isoforms of cyclin E. The increase in functional activity of cyclin E/cdk2 is likely due to a number of factors. First, an absolute increase in the amount of cyclin E expression. Although not directly tested in this paper, we have previously described in mammary epithelial cells, that overexpression of full length cyclin E does not confer the same increase in cyclin E function seen with expression of the T1 and T2 cyclin E isoforms (Wingate et al., 2003). Therefore, it appears that the LMW isoforms have unique properties that enhance function independent of absolute cyclin E protein level. In this context, it is of interest to note that in the overwhelming number of the tumors we have tested by western blot analysis, cyclin E deregulation is invariably associated with the appearance of the LMW isoforms.

Second, since we do not see an increase in the levels of cdk2 in cells transfected with LMW cyclin E, it is also likely that there is more efficient binding between the LMW cyclin E and cdk2 leading to increased activity. Third, although the absolute ratio of cyclin E to CKIs appears to be increased in our MDAH-2774 clones overexpressing T1, the LMW cyclin E also appears to be

more resistant to p21 and p27 mediated inhibition. This resistance is also suggested by the data from the ovarian tumor samples which demonstrate increased cyclin E/cdk2 kinase function despite levels of p21 and p27 that are comparable to or higher than control tumors expressing only the full length cyclin E protein. Therefore, the increase in functional activity seen with LMW cyclin E overexpression is likely multi-factorial and includes more efficient binding to cdk2 and resistance to inhibition by CKIs.

Our data also demonstrates that the resistance to G1 arrest and increase in S-phase fraction in cells expressing LMW cyclin E isoforms has important implications for chemotherapeutic regimens. Agents such as 5-FU, gemcitabine and interferon-alpha that mediate at least some of their effect through arrest in the G1 phase (Cappella et al., 2001; Johnson et al., 1997; Sangfelt et al., 1999) might be expected to have reduced efficacy. Conversely, given the mitogenic effect of cyclin E and the increase in the proportion of cells in S-phase in cyclin E overexpressing cells, drugs that cause DNA damage may have greater efficacy. In ovarian carcinoma, the response to cisplatin therapy has been linked to the proliferative rate of the tumor (Itamochi et al., 2002; Kolfschoten et al., 2000); tumor cells with high S-phase have increased sensitivity to cisplatin treatment (Itamochi et al., 2002; Kolfschoten et al., 2000) . Additionally *in vitro* pharmacologic studies have demonstrated that cyclin E overexpression enhances the cytotoxicity of cisplatin/taxol combination therapy in a panel of different solid tumor cell lines (Smith & Seo, 2000) and that abrogation of the G1/S checkpoint may be involved in increasing the efficacy of cisplatin in ovarian cancer cells (Pestell et al., 2000). Our results, using an isogenic ovarian tumor model of stable cyclin E overexpression with consequent G1 checkpoint abrogation, support these previous observations.

In summary, expression of the LMW isoforms of cyclin E in ovarian tumor cells results in an increase in function of the cyclin E/cdk2 kinase complex with subsequent biologic alterations suggestive of more aggressive phenotype. Tumor cells overexpressing cyclin E also show resistance to G1 arrest, but are more susceptible to the effect of drugs that target the S-phase such as cisplatin. These findings offer insights into the potential mechanisms that underlie the poor clinical outcomes of patients with cyclin E overexpressing tumors, and also underscore the importance of rationale targeting of chemotherapeutic agents based on the inherent biologic properties of the tumors.

Conclusions

As presented in detail in this report-we have addressed the first 3 tasks of the grant application in the last 24 months.

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